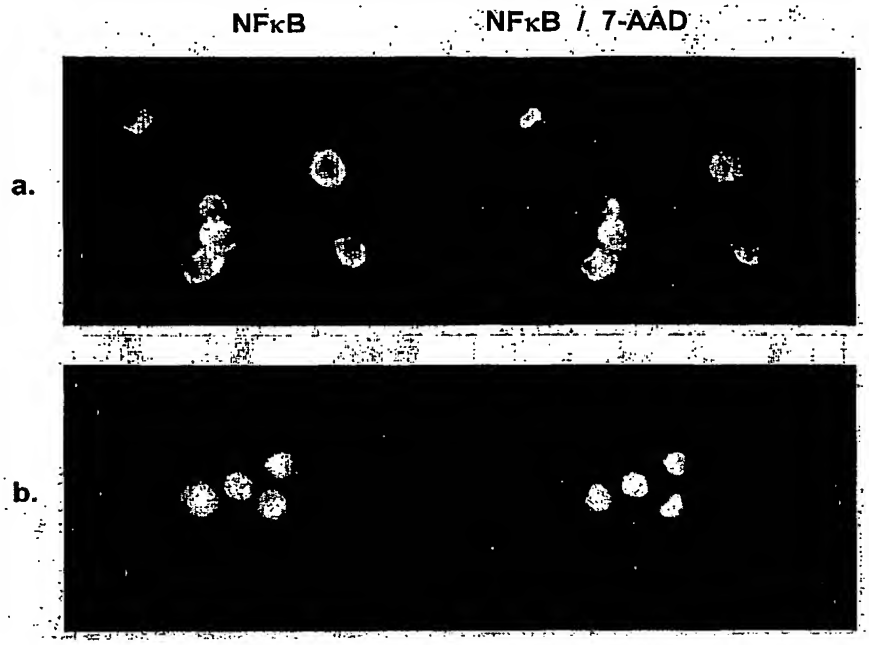


Figure 1: Visualization of NFκB Nuclear Translocation in A549 Cells Using Immunofluorescence Microscopy

TNF-α and IL-1β stimulation initiates a signaling cascade that results in the translocation of NFκB from the cytoplasm to the nucleus of the adherent human carcinoma cell line A549 cells. Untreated A549 cells (a) and A549 cells treated with TNF-α (2 ng/ml) and IL-1β (10 pg/ml) for 1 hour (b) were trypsinized and probed for NFκB expression and nuclear morphology. Briefly, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% triton and incubated with mouse anti-NFκB (p65) + Alexa Fluor® 488 donkey anti-mouse IgG. Cells were washed and resuspended in 1% paraformaldehyde containing 7-AAD, then mixed with an equal volume of antifade and visualized on slides using a Nikon Eclipse E600 fluorescence microscope equipped with bandpass filters appropriate for FITC (535/40 nm) and 7-AAD (630/60 nm) fluorescence. NFκB images in grey are depicted on the left. NFκB (green) / 7-AAD (red) composite images on the right demonstrate the nuclear localization of NFκB following TNF-α / IL-1β treatment.

Fig. 1

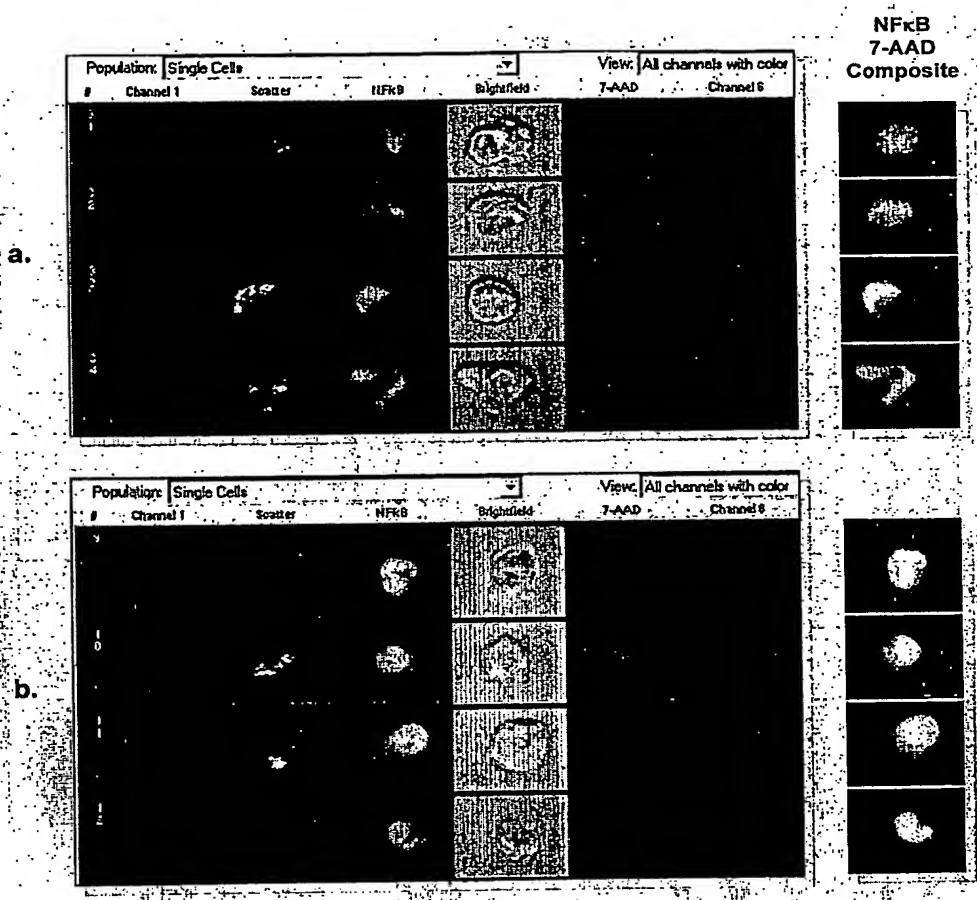


Figure 2: Visualization of NFκB Nuclear Translocation in A549 Cells Using ImageStream Multispectral Imagery

Untreated (a) and TNF-α / IL-1β treated (b) A549 cells were prepared as described in Figure 1 and imaged in flow using the ImageStream 100 instrument. Hydrodynamically focused cells were illuminated from the side with a 488 nm laser and from behind with a brightfield source that was passed through a 577/10 bandpass filter. Four out of a possible six images were used in this experiment. Laser side scatter (pseudocolored blue), NFκB fluorescence (green), brightfield, and 7-AAD fluorescence (red) from the cells were spectrally decomposed and imaged simultaneously onto spatially separated channels on a CCD detector. NFκB (green) / 7-AAD (red) composite images on the right demonstrate the nuclear localization of NFκB following TNF-α / IL-1β treatment.

Fig. 2

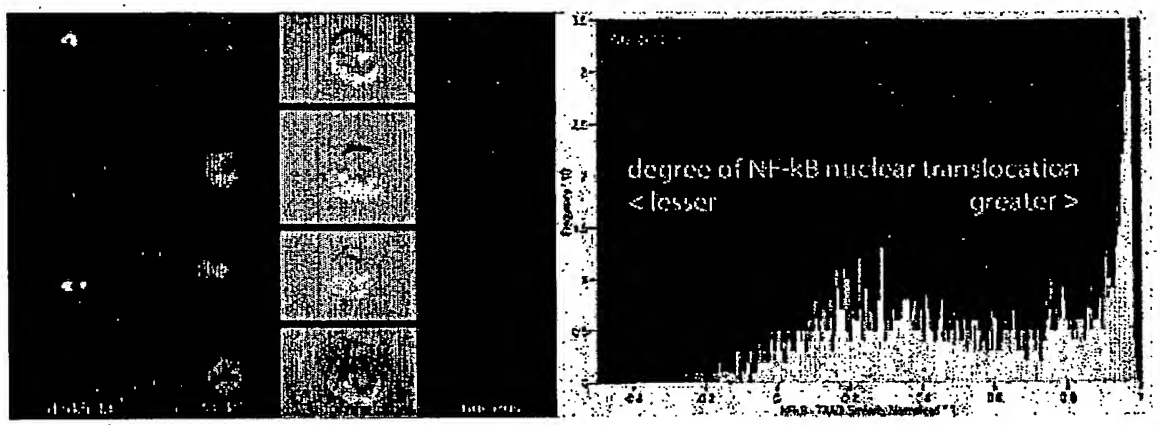


Figure 2C. NF-kB Nuclear Translocation in Immune Cells

The data (above left) show cells imaged simultaneously in darkfield, green fluorescence, brightfield, and red fluorescence. The sample consisted of a monocytic cell line stained with an antibody against the NF -kB transcription factor (green) as well as a nuclear stain (red). Cells treated with lipo-polysaccharide (image rows 2-4) exhibit translocation of NF-kB from the cytoplasm to the nucleus while untreated cells lack NF -kB in the nuclear compartment (top row) . A statistical analysis of imagery from 6616 cells quantitatively characterizes the degree of NF -kB nuclear translocation in the sample. Amnis' ImageStream platform is the only cell analysis technology that can perform this valuable assay on immune cells in suspension.

Fig. 2C

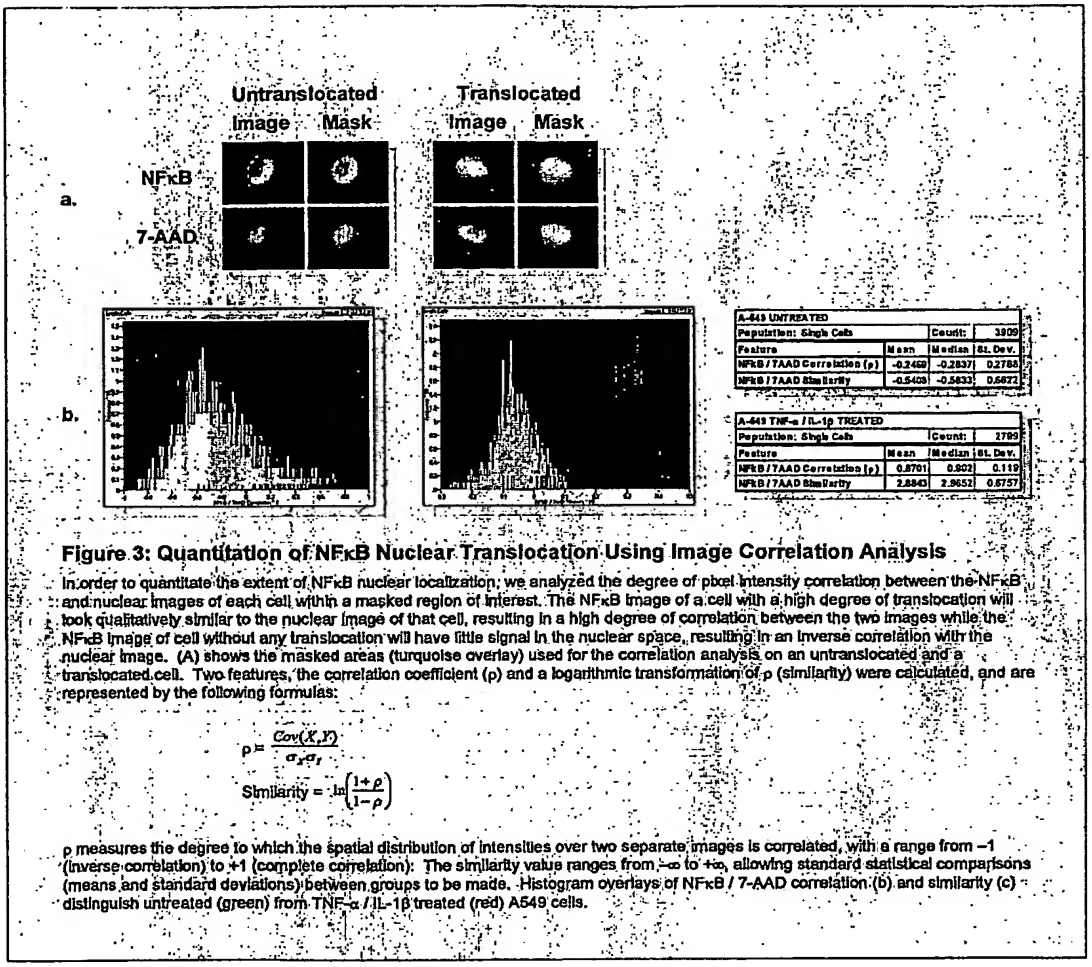


Figure 3: Quantitation of NFκB Nuclear Translocation Using Image Correlation Analysis

In order to quantitate the extent of NFκB nuclear localization, we analyzed the degree of pixel intensity correlation between the NFκB and nuclear images of each cell within a masked region of interest. The NFκB image of a cell with a high degree of translocation will look qualitatively similar to the nuclear image of that cell, resulting in a high degree of correlation between the two images while the NFκB image of cell without any translocation will have little signal in the nuclear space, resulting in an inverse correlation with the nuclear image. (A) shows the masked areas (turquoise overlay) used for the correlation analysis on an untranslocated and a translocated cell. Two features, the correlation coefficient (ρ) and a logarithmic transformation of ρ (similarity) were calculated, and are represented by the following formulas:

$$\rho = \frac{Cov(X,Y)}{\sigma_x \sigma_y}$$

$$\text{Similarity} = \ln\left(\frac{1+\rho}{1-\rho}\right)$$

ρ measures the degree to which the spatial distribution of intensities over two separate images is correlated, with a range from –1 (inverse correlation) to +1 (complete correlation). The similarity value ranges from $-\infty$ to $+\infty$, allowing standard statistical comparisons (means and standard deviations) between groups to be made. Histogram overlays of NFκB / 7-AAD correlation (b) and similarity (c) distinguish untreated (green) from TNF-α / IL-1β treated (red) A549 cells.

Fig. 3

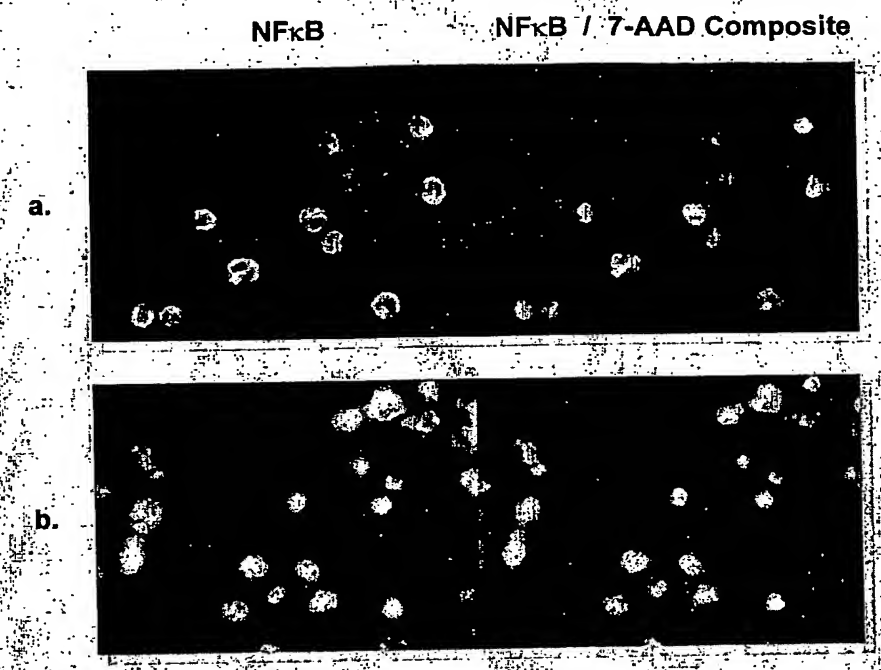


Figure 4: Visualization of NFκB Nuclear Translocation in THP-1 Cells Using Immunofluorescence Microscopy

LPS stimulation initiates a signaling cascade that results in the translocation of NFκB from the cytoplasm to the nucleus of the non-adherent human monocyte cell line THP-1. Untreated THP-1 cells (a) and THP-1 cells treated with LPS (100 ng/ml) for 1 hour (b) were probed for NFκB expression and nuclear morphology. Briefly, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% triton, and incubated with mouse anti-NFκB (p65) + Alexa Fluor® 488 donkey anti-mouse IgG. Cells were washed and resuspended in 1% paraformaldehyde containing 7-AAD, then mixed with an equal volume of antifade and visualized on slides using a Nikon Eclipse E600 fluorescence microscope equipped with bandpass filters appropriate for FITC (535/40 nm) and 7-AAD (630/60 nm) fluorescence. Note the relatively thin band of cytoplasm in the untranslocated NFκB images characteristic of this monocytic cell line.

Fig. 4

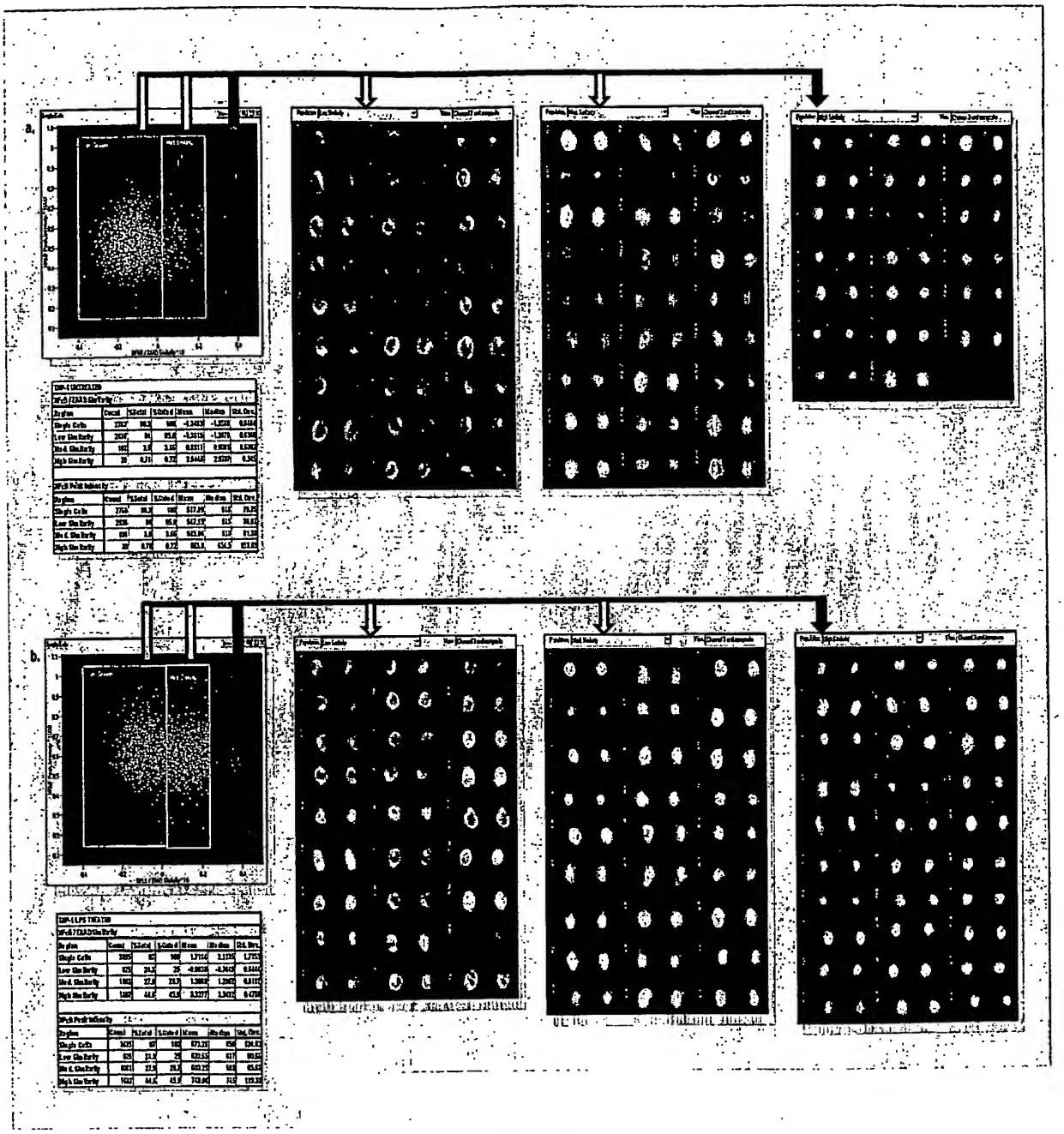


Fig. 5

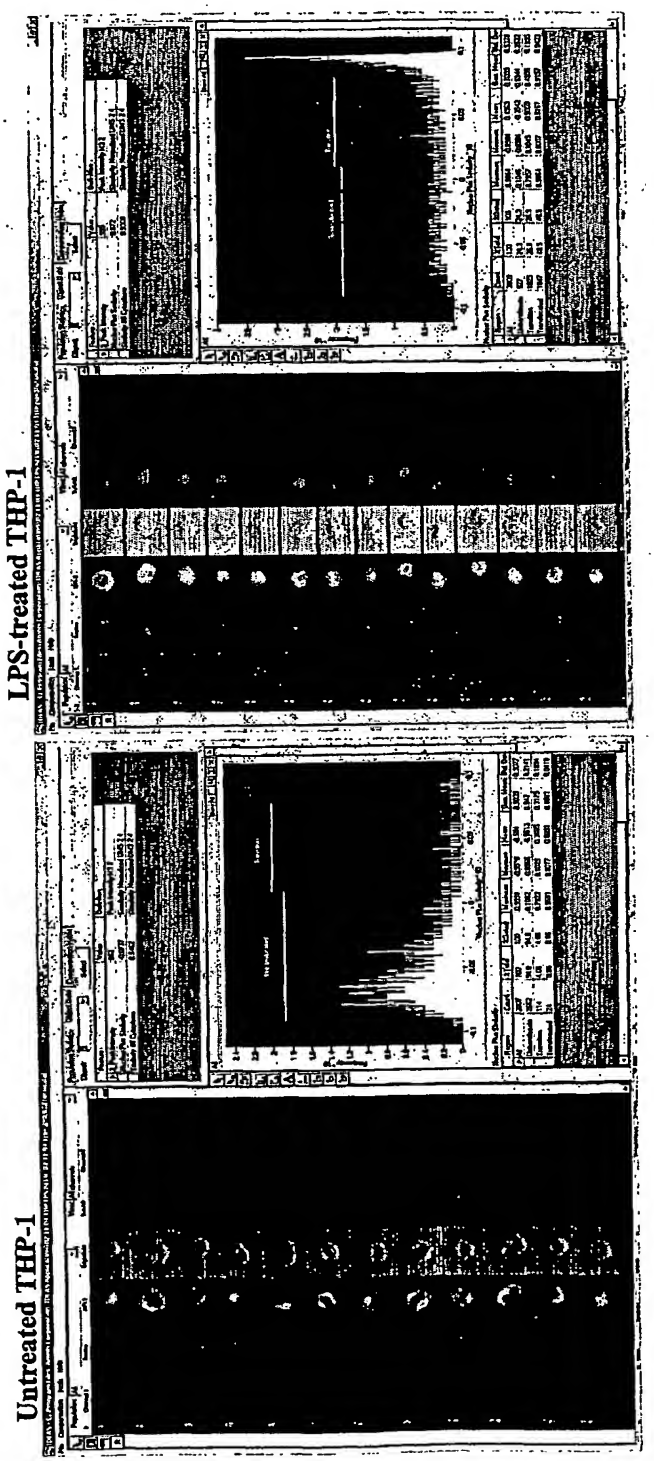


Fig. 6

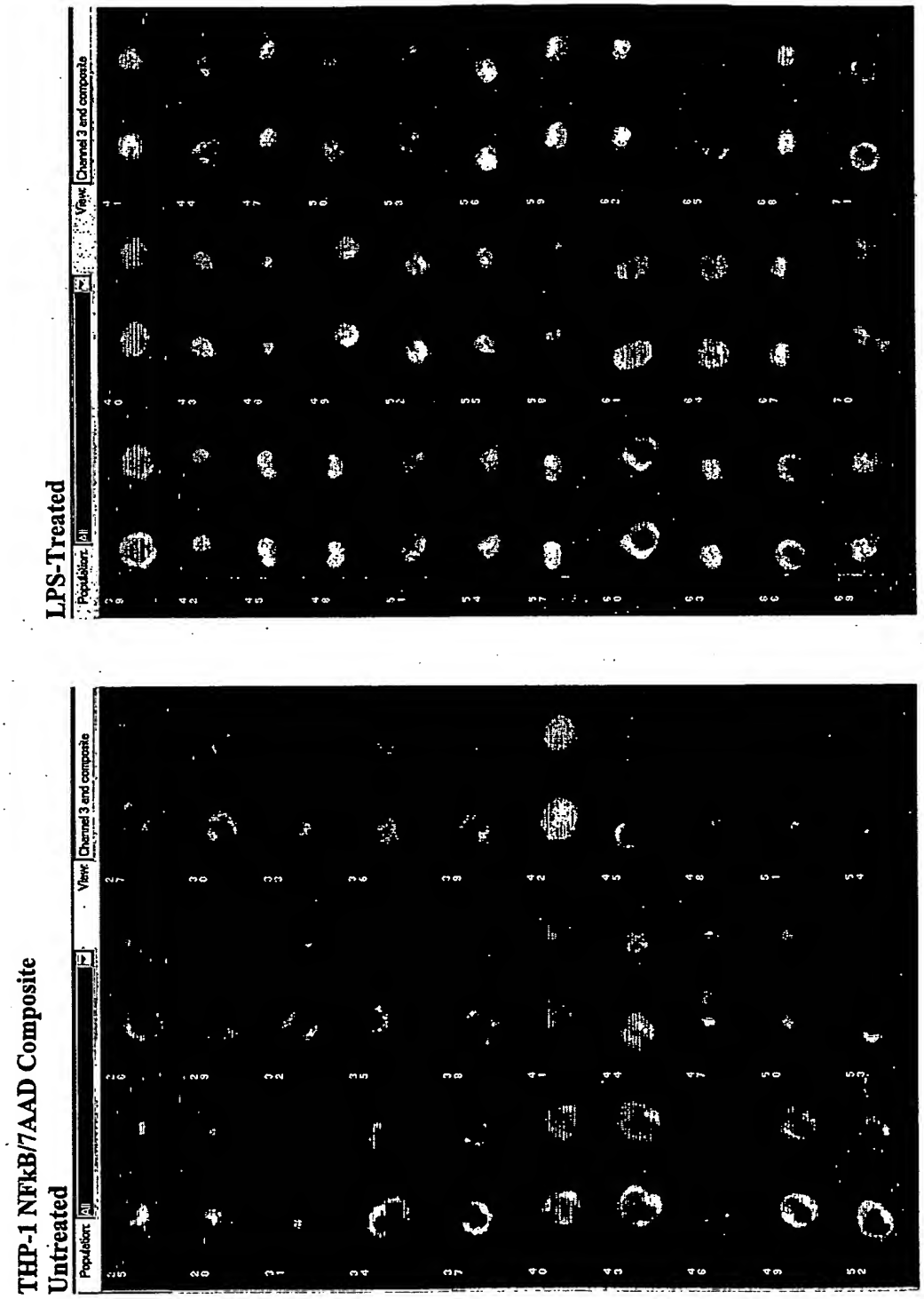
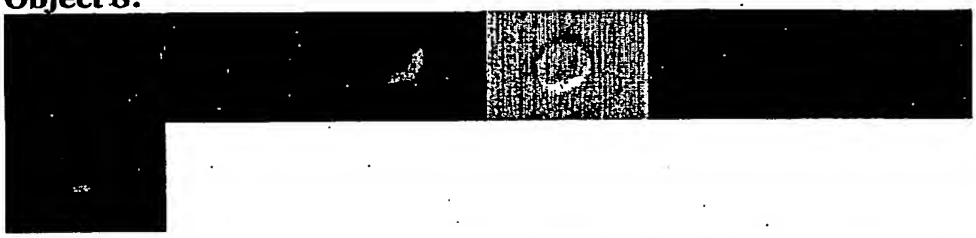
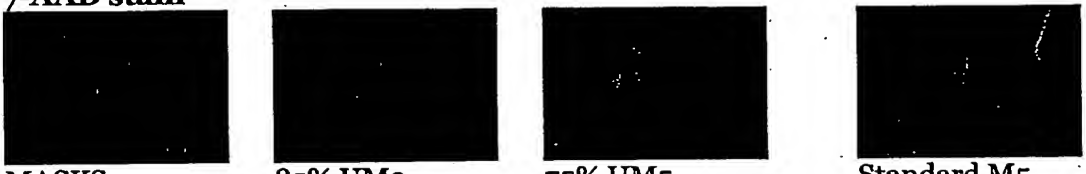

THP-1 NFkB/7AAD Composite
Untreated
Population: All
View: Channel 3 and composite
LPS-Treated
Population: All
View: Channel 3 and composite

Fig. 7

Object 8:



7-AAD stain



MASKS: 85% UM3 75% UM5 Standard M5

NFκB stain

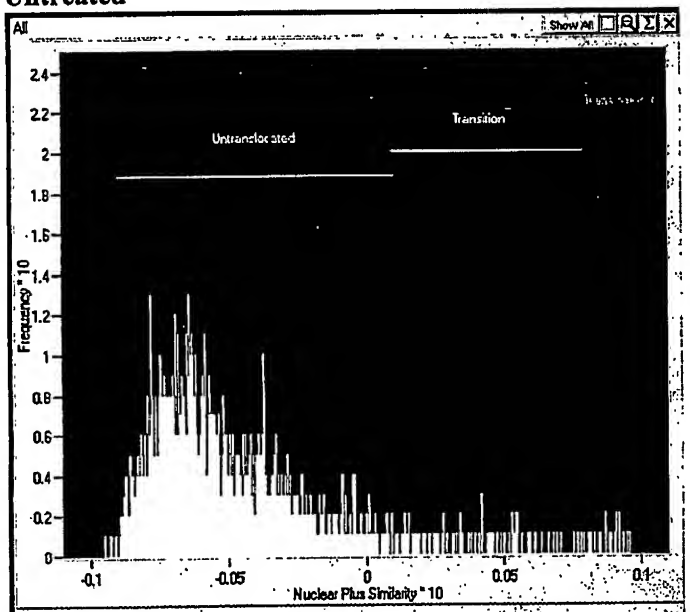
MASKS: 85% UM3 75% UM5

	Feature	Value	Definition
▶	3_Peak Intensity	571	Peak Intensity M3 2
	Nuclear Plus Similarity	-0.155	Similarity Normalized UM5 2 4
	Similarity 85 Cytoplasm	0.5003	Similarity Normalized UM3 2 4

Fig. 8

COMPARTMENTAL CORELATION FEATURE:

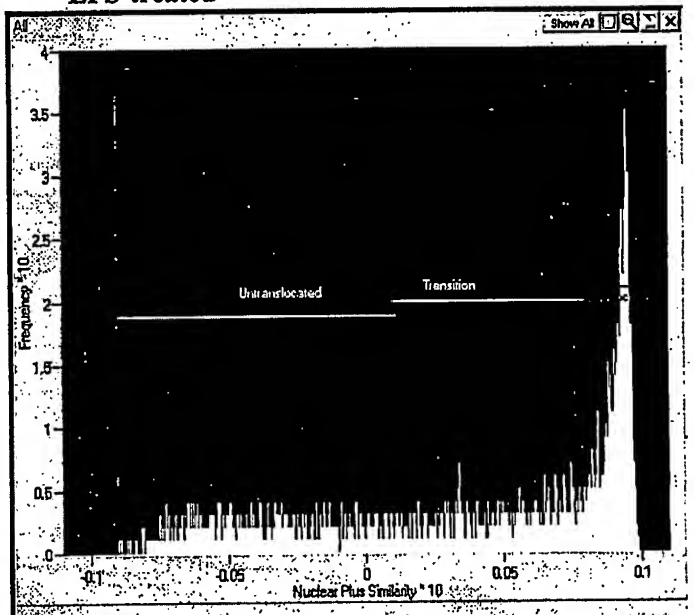
Untreated



Nuclear Plus Similarity

Region	Count	%Total	Mean	Std. Dev.	Median	Variance	CV
All	2807	100	-0.504	0.3077	-0.5875	0.0947	61.06
Untranslocated	2662	94.8	-0.5513	0.2141	-0.5966	0.0458	38.83
Transition	114	4.06	0.3685	0.1894	0.3457	0.0359	51.39
Translocated	24	0.86	0.8891	0.0418	0.8951	0.0018	4.705

LPS-treated



Nuclear Plus Similarity

Region	Count	%Total	Mean	Std. Dev.	Median	Variance	CV
All	3809	100	0.4969	0.5378	0.7818	0.2892	108.23
Untranslocate	927	24.3	-0.3542	0.2532	-0.3677	0.0641	71.47
Transition	1025	26.9	0.5059	0.1995	0.5378	0.0398	39.44
Translocated	1847	48.5	0.9167	0.0423	0.9286	0.0018	4.6122

Median Compartmental Corelation Feature:

Untranslocated = -0.5966 +/- 0.2141

Translocated = 0.9286 +/- 0.0423

Difference of 1.5252

Fig. 9

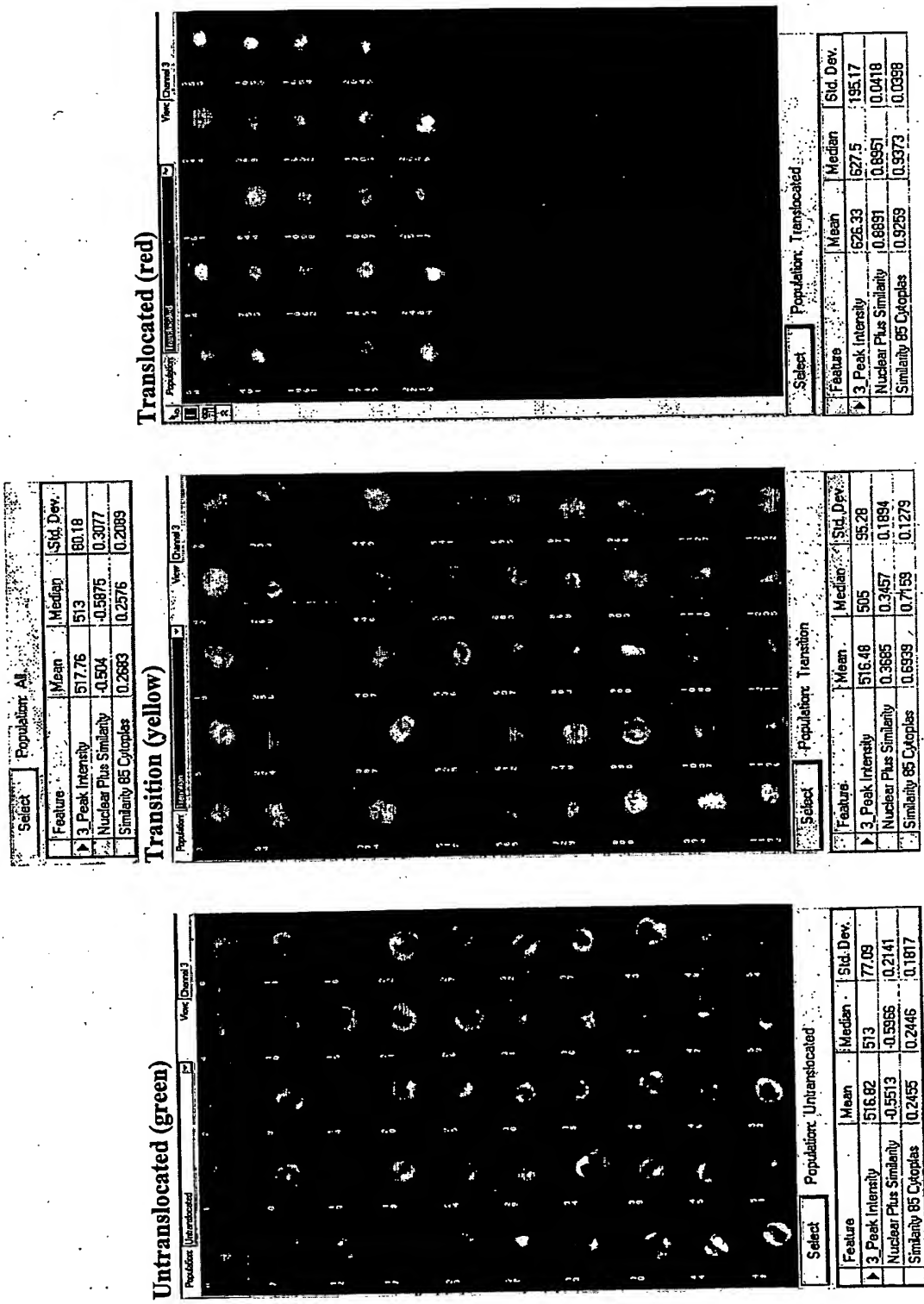

Untranslocated (green)
Transition (yellow)
Translocated (red)
Select
Population: All
Feature
Mean
Median
Std. Dev.
3_Peak Intensity
517.76
513
60.18
Nuclear Plus Similarity
-0.504
-0.5875
0.3077
Similarity 85 Cytoplas
0.2663
0.2576
0.2083
Population: Untranslocated
516.82
513
77.09
-0.5513
-0.5955
0.2141
0.2455
0.2446
0.1817
Population: Transition
516.48
505
95.28
0.3685
0.3457
0.1894
0.6939
0.7153
0.1279
Population: Translocated
626.33
627.5
195.17
0.8891
0.8951
0.0418
0.9269
0.9373
0.0388

Fig. 10A

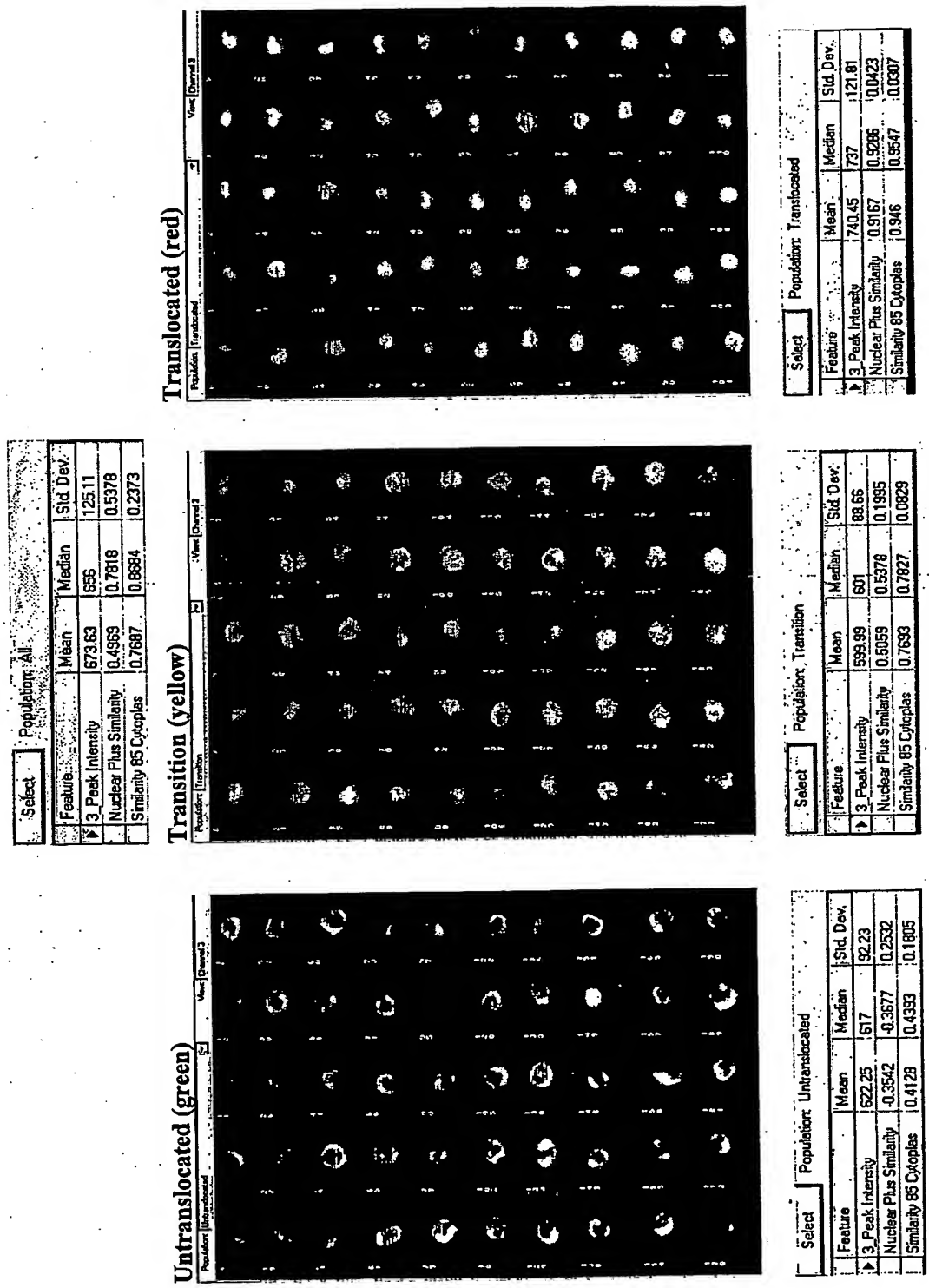

Untranslocated (green)
Transition (yellow)
Translocated (red)
Select
Population: All
Feature | Mean | Median | Std. Dev.
3_Peak Intensity | 673.63 | 656 | 125.11
Nuclear Plus Similarity | 0.4969 | 0.7818 | 0.5378
Similarity 85 Cytoplas | 0.7687 | 0.8684 | 0.2373
Select
Population: Untranslocated
Feature | Mean | Median | Std. Dev.
3_Peak Intensity | 622.25 | 617 | 92.23
Nuclear Plus Similarity | -0.3542 | -0.3677 | 0.2532
Similarity 85 Cytoplas | 0.4128 | 0.4393 | 0.1805
Select
Population: Transition
Feature | Mean | Median | Std. Dev.
3_Peak Intensity | 599.99 | 601 | 88.66
Nuclear Plus Similarity | 0.5059 | 0.5378 | 0.1995
Similarity 85 Cytoplas | 0.7693 | 0.7827 | 0.0829
Select
Population: Translocated
Feature | Mean | Median | Std. Dev.
3_Peak Intensity | 740.45 | 737 | 121.81
Nuclear Plus Similarity | 0.9167 | 0.9286 | 0.0423
Similarity 85 Cytoplas | 0.946 | 0.9547 | 0.0307

Fig. 10B

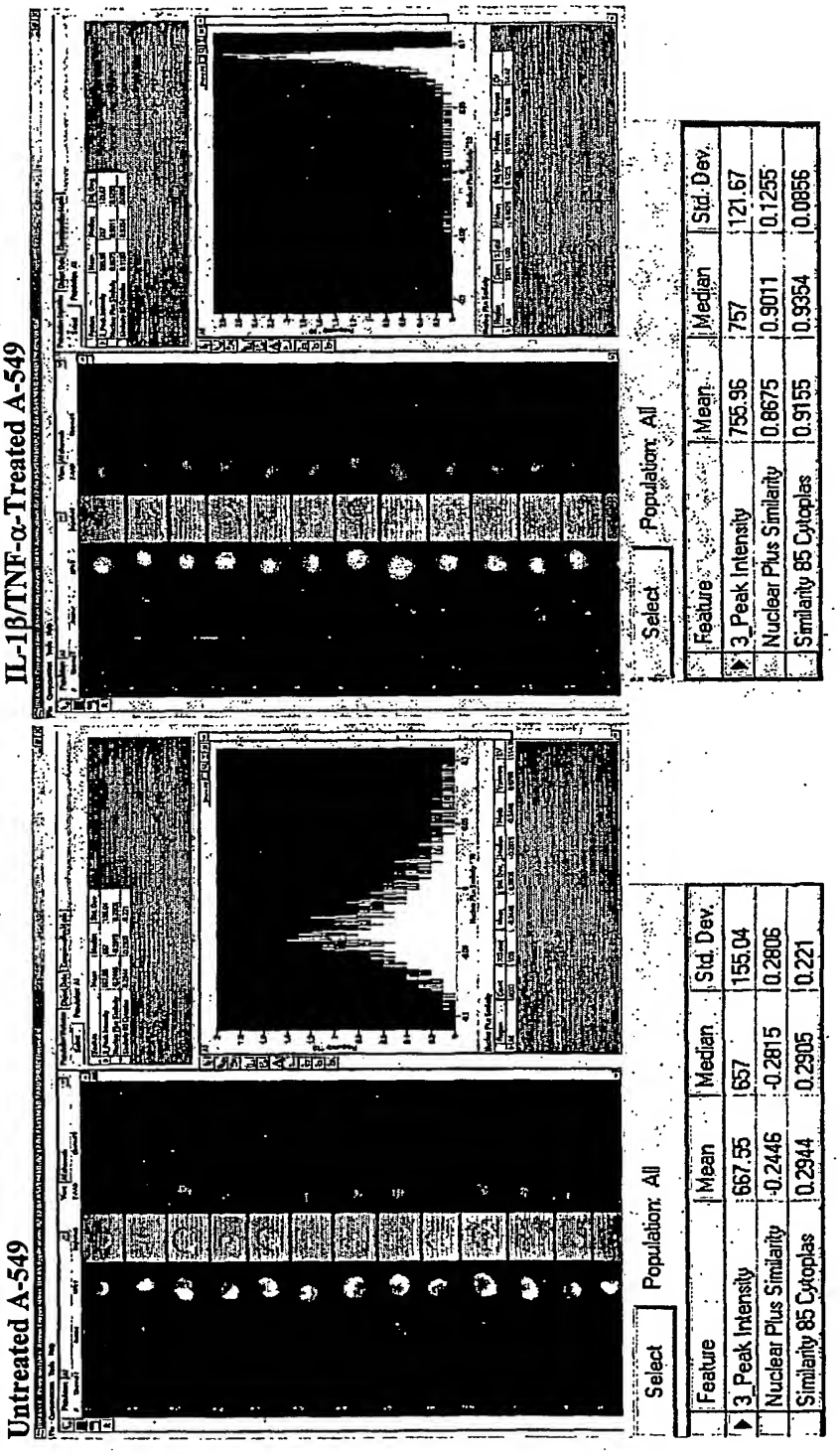

Untreated A-549
IL-1β/TNF-α-Treated A-549
Select
Population: All
Feature | Mean | Median | Std. Dev.
3_Peak Intensity | 667.55 | 657 | 155.04
Nuclear Plus Similarity | -0.2446 | -0.2815 | 0.2806
Similarity 85 Cytoplas | 0.2944 | 0.2905 | 0.221
Select
Population: All
Feature | Mean | Median | Std. Dev.
3_Peak Intensity | 755.96 | 757 | 121.67
Nuclear Plus Similarity | 0.8675 | 0.9011 | 0.1255
Similarity 85 Cytoplas | 0.9155 | 0.9364 | 0.0856

Fig. 11

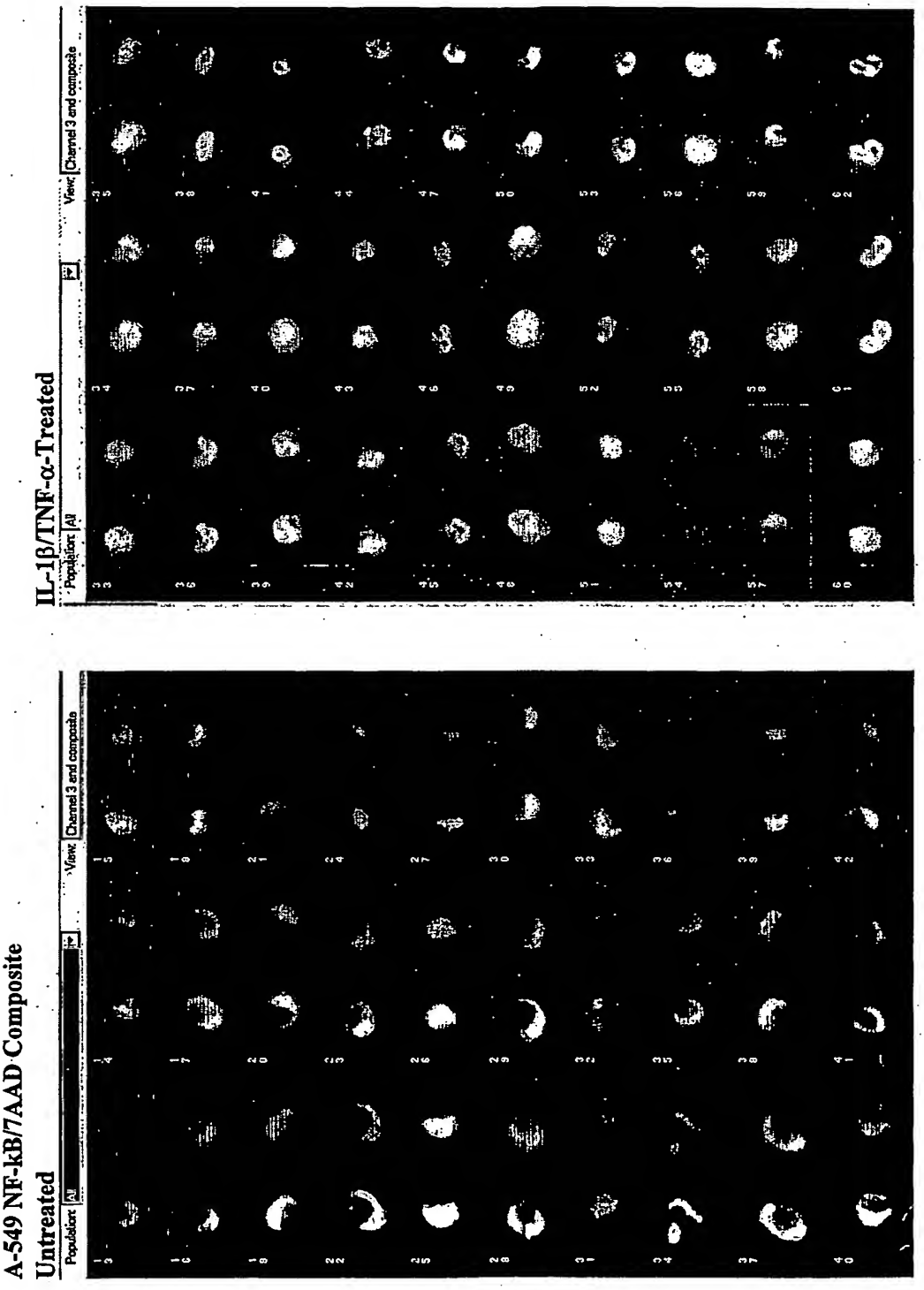
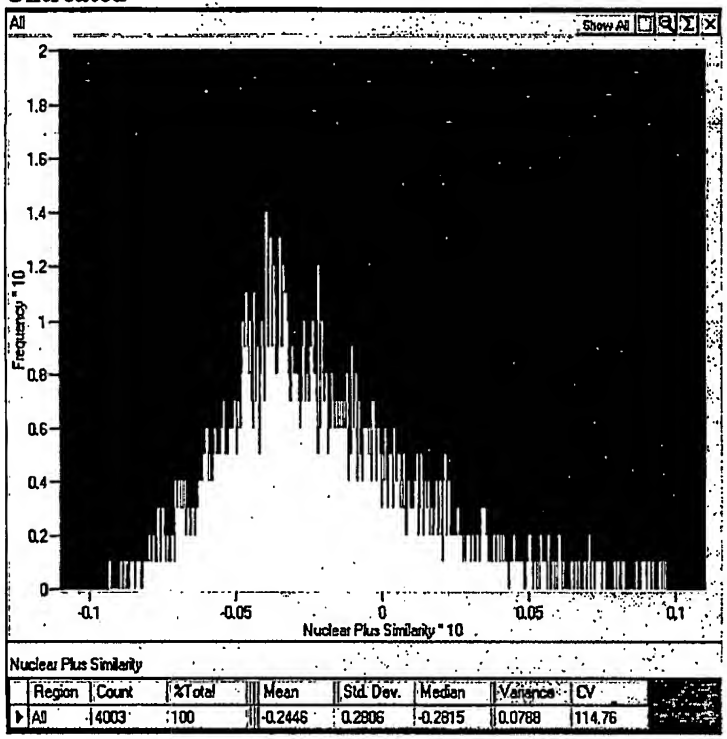

A-549 NF-kB/7AAD Composite
Untreated
Population: All
View: Channel 3 and composite
IL-1β/TNF-α-Treated
Population: All
View: Channel 3 and composite

Fig. 12

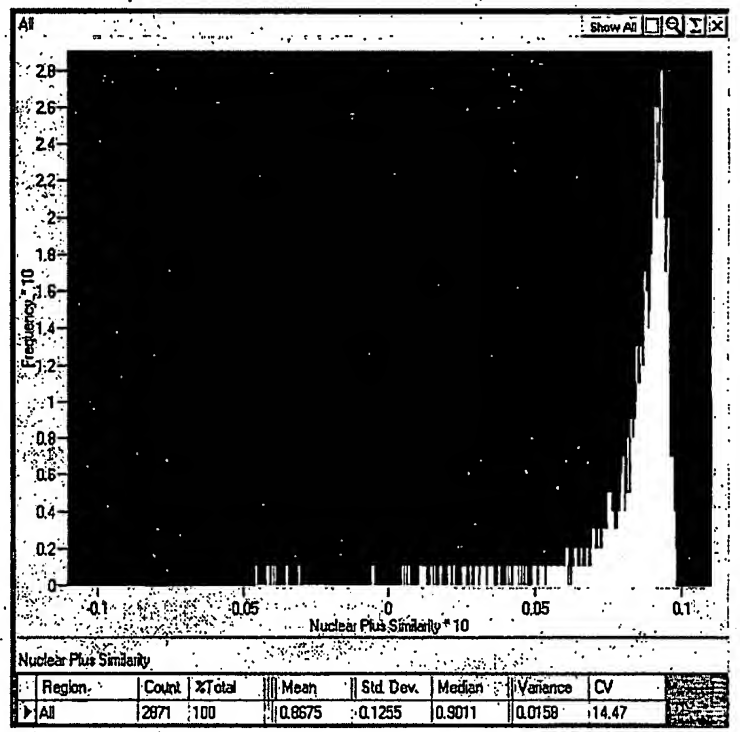
Compartmental Corelation Feature: A-549 Cells

Untreated



Region	Count	%Total	Mean	Std. Dev.	Median	Variance	CV
All	4003	100	-0.2446	0.2806	-0.2815	0.0788	114.76

IL-1β/TNF-α-Treated



Region	Count	%Total	Mean	Std. Dev.	Median	Variance	CV
All	2871	100	0.8675	0.1255	0.9011	0.0158	14.47

Median Compartmental Corelation Feature:

-2815 +/- 0.2806

0.9011 +/- 0.1255

Difference of 1.1826

Fig. 13